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Chemotherapeutic agents used in the treatment of breast cancer produce their cytotoxic effects by creating DNA damage. Estrogen (ER) and retinoic acid receptors (RAR) are members of a family of ligand dependent transcription factors. ER, RAR, and BRCA1 require CREB binding protein (CBP) to activate target gene transcription. The application proposed a new mechanism by which ER and RAR regulate BRCA1 mediated DNA repair via CBP. In the second year of the project, we determined that RARα overexpression in ER negative breast cancer cell lines enhances the deleterious effects of RA on DNA damage induced apoptosis. Treatment with the DNA methyltransferase inhibitor ADC failed to induce additional BRCA1 expression in ER negative breast cancer cell lines. A novel BRCA1 mutant protein repressed expression of a number of double strand break repair proteins in the Rad and XRCC groups. However, both T47D and MDA-MB-468 clones expressing the novel BRCA1 mutant protein were more resistant to the effects of etoposide treatment. These results may be due to the pronounced cell cycle inhibitory effect of the BRCA1 mutant protein, thereby rendering the slower dividing cells less sensitive to the topoisomerase inhibitor.

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INTRODUCTION

BRCA1, DNA Repair, and Breast Cancer. Breast cancer is one of the leading causes of death in women. The disease and its consequences are a significant cause of morbidity and mortality (Russo, 2000). Surgical removal of the tumor followed by radiotherapy is the therapeutic mainstay for early disease; however mastectomy with axillary lymph node dissection and chemotherapy is required for disseminated breast cancer. Inactivating mutations in the tumor suppressor BRCA1 have been discovered in familial forms of the disease and are associated with significantly increased risk of developing breast cancer (Yang and Lippman, 1999). The BRCA1 gene encodes a protein shows no significant similarity to previously described proteins with the exception of a RING zinc finger motif in the amino terminus and carboxyl terminal repeats (Bertwistle and Ashworth, 1998). The carboxyl terminal repeats are found in a range of proteins involved in DNA repair (Koonin et al., 1996; Callebaut and Mornon, 1997). BRCA1 has been shown to induce expression of the DNA damage response gene GADD45 (MacLachlan et al., 2000). Additionally, BRCA1 functionally associates with Rad51 protein which is involved in double strand break repair (Scully et al., 1997). This evidence suggests an important role for BRCA1 in DNA repair and maintaining genome integrity (Kinzler and Vogelstein, 1997; Brugarolas and Jacks, 1997). BRCA1 is involved in repair of double strand breaks induced by chemotherapy drugs (Husain et al., 1998). A number of chemotherapeutic agents used in the treatment of breast cancer produce their cytotoxic effects by creating DNA damage (Hoeijmakers, 2001).

Nuclear Hormone Receptors, Coactivators, and BRCA1. Among the most important nuclear hormone receptors expressed by breast cancer cells are those for estrogen and retinoic acid (Russo and Russo, 1998). Estrogens such as 17-β-estradiol (E2) have been shown to dramatically enhance proliferation of mammary gland epithelium (Huseby et al., 1984). In contrast, a number of natural and synthetic retinoids have been shown to inhibit proliferation of these cells and have been used as chemotherapy drugs in the treatment of breast cancer (Li et al., 1999). Estrogen receptors (ER) and retinoic acid receptors (RAR) are members of a family of ligand dependent transcription factors that include steroid, thyroid, and vitamin D receptors (Mangelsdorf et al., 1995). Both ER and RAR have functional domains for DNA binding, ligand binding, dimerization, and transcriptional activation. ER and RAR require coactivator proteins such as CREB binding protein (CBP) to activate target gene transcription. CBP interacts with ER and RAR in their ligand bound conformation to induce gene expression (Chakravarti et al., 1996). CBP has histone acetyltransferase activity, allowing for histone disassembly and transcriptional activation (Ogryzko et al., 1996). CBP has also been shown to interact with and enhance the function of BRCA1 (Pao et al., 2000).

A New Role for Estradiol and Retinoic Acid in BRCA1 Mediated DNA Repair. While the effects of E2 and RA on proliferation of human breast cancer cells have been known for many years, no studies have suggested a role for these hormones in DNA repair. The original application proposed a new mechanism by which ER and RAR regulate BRCA1 mediated DNA repair via CBP. This model may ultimately predict which breast cancers will respond to the inclusion of retinoids in the chemotherapy regimen.

BODY OF REPORT

In the second year of the funded application (April 2003-April 2004), we have made substantial progress towards accomplishing Task 2 as outlined in the Statement of Work. We have created a large number of stable clones overexpressing RARα in the ER negative MDA-MB-231 and MDA-MB-468 cell lines. We have also created stable clones in T47D and MDA-MB-468 cells expressing a novel BRCA1 mutant. The characterization of these clones is presented below. We are also creating stable clones from ER negative cell lines which overexpress ERα as part of Task 2a; these clones are still in the expansion phase. We expect to begin the characterization of these clones in May 2004.

As part of Task 2b, we have created stable clones overexpressing RARα using ER negative MDA-MB-231 and MDA-MB-468 cell lines. We then determined levels of DNA damage induced cell death in this clones treated with E2 or RA followed by the topoisomerase inhibitor etoposide. We used 30 μg/ml etoposide to induce double strand DNA breaks; control cultures were treated with 0.1% DMSO vehicle. Etoposide treatment of control clones resulted in 50% apoptotic cells by 24 hours after addition to the culture medium. Etoposide treatment of RARα overexpressing clones showed an increased the fraction of apoptotic cells (60%) even in the absence of RA pretreatment. Prior treatment with 100 nM RA increased the fraction of apoptotic cells in etoposide treated cultures to 70% in control clones and 80% in RARα overexpressing clones. Pretreatment with E2 failed to exert a protective effect in these ER negative clones. We concluded that RARα overexpression increased the apoptotic effects of etoposide induced DNA double strand break damage which was further augmented by RA treatment.

A previous study suggested that decreased BRCA1 expression in some ER negative breast cancers was due to methylation of the gene promoter region (Niwa et al., 2000). To determine if this mechanism was important in the regulation of BRCA1 expression in breast cancer cell lines, we treated the ER negative lines MDA-MB-213, MDA-MB-468, SKBR3, and Hs578T with 1 μM azadeoxycytidine (ADC) in culture for 8 days as part of Task 2c. However, ADC treatment failed to induce BRCA1 expression in any of these cell lines. The recruitment of CBP to RARα by RA treatment was similar in both ADC and vehicle treated cultures. GADD45 expression in response to etoposide induced DNA damage also was largely unaffected by ADC treatment. We concluded that promoter methylation is likely not the major mechanism resulting in decreased BRCA1 expression in human breast cancer cell lines (see Conclusions).

Also in Task 2c, we investigated the function of a novel BRCA1 mutant in modifying the hormone regulated DNA damage response. We have characterized a novel BRCA1 mutant which lacks the carboxyl terminal 276 amino acids containing the BRCT repeats believed to be involved in DNA repair (Li et al., 1999). We created stable clones expressing the BRCA1 mutant in ER positive T47D cells and the ER negative MDA-MB-468 line. The BRCA1 mutant inhibited cellular proliferation of both T47D and MDA-MB-468 clones. The BRCA1 mutant clones proliferated 30% more slowly than

control cells (doubling time 29 vs. 38 hours). We examined expression of double strand break and mismatch repair proteins in these clones in response to etoposide. The effects of the BRCA1 mutant were different in the two cell lines. Expression of the BRCA1 target gene GADD45 was induced 2 fold by etoposide in MDA-MB-468 control clones. However, the BRCA1 mutant repressed GADD45 expression to undetectable levels in these cells. In T47D clones, GADD45 expression did not respond to etoposide treatment but was 2 fold higher in BRCA1 mutant cells. Expression of a number of double strand break repair proteins of the Rad and XRCC groups expression was induced by etoposide treatment but strongly repressed by the BRCA1 mutant protein. XRCC1 expression was not induced by etoposide treatment but was inhibited by the BRCA1 mutant protein. Expression of the mismatch repair protein XPA was not affected by etoposide treatment or expression of the BRCA1 mutant. However, expression of two other mismatch repair proteins MLH1 and MSH2 were repressed by the BRCA1 mutant in both T47D and MDA-MB-468 clones. These results indicate that the mutant BRCA1 protein represses expression of double strand break repair proteins, which correlates with previous reports of enhanced sensitivity to DNA damage in BRCA1 mutant cells (Zhou et al., 2003).

Expression of the BRCA1 mutant did not alter the effects of E2 and RA treatment on DNA damage induced apoptosis of breast cancer cell lines. Based on the repression of double strand break protein expression by the BRCA1 mutant, we predicted that these clones would be more sensitive to the DNA damaging effects of etoposide. However, both T47D and MDA-MB-468 clones expressing the novel BRCA1 mutant protein were more resistant to the effects of etoposide treatment. After 24 hours treatment, 50% of cells in control cultures had undergone apoptosis as measured by TUNEL assay compared to only 30% of cells expressing the BRCA1 mutant protein. These results may be due to the pronounced cell cycle inhibitory effect of the BRCA1 mutant protein thereby rendering the slower dividing cells less sensitive to the topoisomerase inhibitor (see Conclusions).

KEY RESEARCH ACCOMPLISHMENTS

Task 2a

We have created and characterized a large number of stable clones expressing four different experimental and control constructs in multiple human breast cancer cell lines. The ERα stable clones are in the last group to be characterized. These clones are in the expansion phase and we expect to begin their characterization in May 2004.

Task 2b

1. Etoposide treatment of MDA-MB-231 and MDA-MB-468 control clones resulted in 50% apoptotic cells by 24 hours after addition to the culture medium.
2. Etoposide treatment of MDA-MB-231 and MDA-MB-468 RARα overexpressing clones showed an increased the fraction of apoptotic cells (60%) even in the absence of RA pretreatment.
3. Prior treatment with 100 nM RA increased the fraction of apoptotic cells in etoposide treated cultures to 70% in control clones and 80% in RARα overexpressing clones.
4. Pretreatment with E2 failed to exert a protective effect in these ER negative clones.

Task 2c

1. ADC treatment failed to induce BRCA1 expression in ER negative breast cancer cell lines.
2. The recruitment of CBP to RARα by RA treatment was similar in both ADC and vehicle treated cultures.
3. GADD45 expression in response to etoposide induced DNA damage also was largely unaffected by ADC treatment.
4. The BRCA1 mutant clones proliferated 30% more slowly than control cells (doubling time 29 vs 38 hours).
5. Expression of the BRCA1 target gene GADD45 was induced 2 fold by etoposide in MDA-MB-468 control clones. However, the BRCA1 mutant repressed GADD45 expression to undetectable levels in these cells. In T47D clones, GADD45 expression did not respond to etoposide treatment but was 2 fold higher in BRCA1 mutant cells.
6. Expression of a number of double strand break repair proteins of the Rad and XRCC groups expression was induced by etoposide treatment but strongly repressed by the BRCA1 mutant protein. Expression of two mismatch repair proteins MLH1 and MSH2 were also repressed by the BRCA1 mutant in both T47D and MDA-MB-468 clones.
7. Both T47D and MDA-MB-468 clones expressing the novel BRCA1 mutant protein were more resistant to the effects of etoposide treatment. After 24 hours treatment, 50% of cells in control cultures had undergone apoptosis as measured by TUNEL assay compared to only 30% of cells expressing the BRCA1 mutant protein.

REPORTABLE OUTCOMES

Not applicable

CONCLUSIONS

In the second year of the funded application we have demonstrated that RARα overexpression in ER negative breast cancer cell lines enhances the deleterious effects of RA on DNA damage induced apoptosis. These data together with that from the first year of the funded application indicate that recruitment of CBP to RARα inhibits the BRCA1 mediated DNA damage response in breast cancer cell lines.

A previous study suggested that decreased BRCA1 expression in some ER negative breast cancers was due to methylation of the gene promoter region (Niwa et al., 2000). However, treatment with the DNA methyltransferase inhibitor ADC failed to induce additional BRCA1 expression in our ER negative breast cancer cell lines. The recruitment of CBP to RARα by RA treatment was similar in both ADC and vehicle treated cultures. GADD45 expression in response to etoposide induced DNA damage also was largely unaffected by ADC treatment. We concluded that promoter methylation is likely not the major mechanism resulting in decreased BRCA1 expression in some ER negative human breast cancer cell lines. However, it will be important to determine directly the degree of BRCA1 promoter methylation in ER positive and negative lines.

We have characterized a novel BRCA1 mutant which lacks the carboxyl terminal 276 amino acids containing the BRCT repeats believed to be involved in DNA repair. The BRCA1 mutant protein repressed expression of a number of double strand break repair proteins in the Rad and XRCC groups. These results indicated that BRCA1 mutations may inhibit DNA repair by decreasing expression of the relevant proteins, which correlates well with enhanced sensitivity to DNA damage in BRCA1 null cells. Additionally, we observed differences between ER positive and ER negative BRCA1 mutant clones regarding regulation of the putative BRCA1 target gene GADD45 in response to etoposide. Expression of the BRCA1 target gene GADD45 was induced by etoposide in MDA-MB-468 control clones, and the BRCA1 mutant repressed GADD45 expression to undetectable levels in these cells. However, in T47D clones GADD45 expression did not respond to etoposide treatment and was higher in BRCA1 mutant cells. These opposite effects may reflect CBP recruitment away from BRCA1 by endogenous ER and RAR in T47D cells as suggested by our data from the first year of the funded application. Expression of mismatch repair proteins was also affected by the BRCA1 mutant protein. While our data from the first year of the funded application indicated that nuclear hormones had little effect on mismatch repair, BRCA1 may have a role in regulating DNA repair when cells are treated with drugs such as cisplatin that activate this particular pathway.

Based on the repression of double strand break protein expression by the BRCA1 mutant, we predicted that these clones would be more sensitive to the DNA damaging effects of etoposide. However, both T47D and MDA-MB-468 clones expressing the novel BRCA1 mutant protein were more resistant to the effects of etoposide treatment. These results may be due to the pronounced cell cycle inhibitory effect of the BRCA1 mutant protein, thereby rendering the slower dividing cells less sensitive to the topoisomerase inhibitor. It will be important to characterize the cell cycle regulatory effects of the BRCA1 mutant,

since this unusual property of the protein produces phenotypic changes that may alter our understanding of the function of this tumor suppressor.

In the third year of the funded application, we will evaluate the proposed model in vivo using primary tumor cells from rat mammary cancers. We will also examine the unexpected cell cycle regulatory functions of the mutant BRCA1 protein. The career development activities detailed in the application will also continue.

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